

# EXHIBIT D

U.S. Serial No. 10/057,288

Shirasugi, Nozumu et al.,

Journal of Immunology,

2002, 169:2677-84

# Prevention of Chronic Rejection in Murine Cardiac Allografts: A Comparison of Chimerism- and Nonchimerism-Inducing Costimulation Blockade-Based Tolerance Induction Regimens<sup>1</sup>

Nozomu Shirasugi, Andrew B. Adams, Megan M. Durham, Aron E. Lukacher, Huaying Xu, Phyllis Rees, Shannon R. Cowan, Matthew A. Williams, Thomas C. Pearson,<sup>2</sup> and Christian P. Larsen<sup>2</sup>

We have previously described a nonirradiation-based regimen combining costimulation blockade, busulfan, and donor bone marrow cells that promotes stable, high level chimerism, deletion of donor-reactive T cells, and indefinite survival of skin allografts in mice. The purpose of the current study is to determine the efficacy of this tolerance regimen in preventing acute and chronic rejection in a vascularized heart graft model and to compare this regimen with other putative tolerance protocols. Mice receiving costimulation blockade (CTLA4-Ig and anti-CD40 ligand) alone or in combination with donor cells enjoyed markedly prolonged heart graft survival and initially preserved histological structure. However, tolerance was not achieved, as evidenced by the eventual onset of chronic rejection characterized by obliterative vasculopathy and the rejection of secondary skin grafts. In contrast, following treatment with costimulation blockade, busulfan, and bone marrow, heart grafts survived indefinitely without detectable signs of chronic rejection or structural damage, even 100 days after placement of a secondary donor skin graft. We detected multilineage chimerism in peripheral blood, spleen, lymph nodes, and thymus, and peripheral deletion of donor-reactive cells was complete by day 90. These findings indicate that only the CD40/CD28 blockade chimerism induction regimen prevents both acute and chronic rejection of vascularized organ transplants. Further testing of these strategies in a preclinical large animal model is warranted. *The Journal of Immunology*, 2002, 169: 2677–2684.

Transplantation has become an accepted treatment for patients with end-stage organ failure. However, clinical success of organ transplantation has been achieved through the use of nonspecific immunosuppressive drugs to inhibit immune responses. These drugs generally need to be given for the life of the transplant and have many potential, direct side effects, as well as increased risk of life-threatening complications such as cardiovascular disease, infections, and malignancies. Moreover, while the long-term outcome of transplantation has improved, it remains inadequate (1). The most common cause of late graft loss is chronic rejection, which is characterized by obliterative vasculopathy (2). The most appealing solution to these problems is the induction of transplantation tolerance, defined as lifelong, donor-specific unresponsiveness without the need for chronic immunosuppression. It has been thought that tolerance induction would not only free patients from the morbidity associated with long-term immunosuppression, but also prevent both acute and chronic allograft rejection. However, recent evidence indicates that tolerance induction may not always prevent chronic rejection (3, 4). Thus, it is imperative that careful studies be

performed to define the effects of specific tolerance induction regimens on the development of chronic rejection.

In recent years, several clinically relevant immunomodulatory or tolerance induction regimens have been reported that incorporate the use of Abs and/or fusion proteins that target the CD28/B7 and CD40/CD154 pathways with or without the concomitant administration of donor cells (splenocytes or bone marrow cells (BMCs)<sup>3</sup>) (5–9). Although the immunological mechanisms underlying the effectiveness of these regimens are incompletely understood, recent advances have suggested that the development of tolerance involves the participation of CD4<sup>+</sup> regulatory T cells that are activated or exert their function in a CTLA-4-dependent manner. Furthermore, evidence suggests that the continued presence of a vascularized allograft can itself play a critical role in tolerance maintenance (10). Although several of these studies have indicated costimulation blockade (CB)-based regimens can inhibit chronic rejection, the duration of follow-up and the tests of the stability of tolerance in these studies provide insufficient data upon which to draw firm conclusions about long-term outcomes (5–7).

The induction of mixed hemopoietic chimerism has been a promising strategy for the induction of robust immunological tolerance for many years (11, 12). Although in the past most chimerism induction regimens required the use of gamma irradiation and/or depletion of the peripheral immune system (13–17), more recently, protocols using either the administration of mega doses of

The Carlos and Marguerite Mason Transplantation Biology Research Center, Departments of Surgery, Emory University School of Medicine, Atlanta, GA 30322

Received for publication February 15, 2002. Accepted for publication June 11, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported in part by Research Grants DK/A140519, CA74364-03, and A144644 from the National Institutes of Health, and by the Engineering Research Centers Program of the National Science Foundation under Award EEC-9731643, as well as by the Carlos and Marguerite Mason Trust. N.S. was financially supported in part by Uehara Memorial Foundation (Tokyo, Japan).

<sup>2</sup> Address correspondence and reprint requests to Dr. Christian P. Larsen or Dr. Thomas C. Pearson, Emory University Woodruff Memorial Building, 1639 Pierce Drive, Atlanta, GA 30322. E-mail address: clarsen@emory.org or tpearson@emory.org

<sup>3</sup> Abbreviations used in this paper: BMC, bone marrow cell; Bu, busulfan; CB, costimulation blockade; CD40L, CD40 ligand; DST, donor splenocyte; MST, median survival time.

donor bone marrow or the use of minimally myelosuppressive recipient conditioning combined with various forms of CB have increased the likelihood that clinically acceptable tolerance induction regimens based on these principles can be devised (18, 19). The strategy that we have used incorporates the CTLA4-Ig fusion protein to target the CD80/CD86-CD28 pathway and MR1, an anti-CD154 mAb to target the CD40-CD40 ligand (CD40L) interactions, and the administration of donor BMCs after conditioning the recipient with a minimally myelosuppressive dose of the chemotherapeutic conditioning agent, busulfan (Bu). This regimen induces high level, stable mixed hemopoietic chimerism and specific deletional tolerance to fully MHC-mismatched allogeneic skin grafts (20).

Due to the limitations of the skin allograft model, our earlier studies were unable to address the important issue of degree of protection conferred from acute cellular infiltration and chronic rejection. Because of the increasing evidence that tolerance may not equate to freedom from chronic rejection, we used the murine cardiac allograft model to compare the ability of this and other promising tolerance induction regimens to protect vascularized allografts from immunologic injury during tolerance induction and to prevent chronic rejection. The need for such studies is underscored by the report by Russell et al. (4) that was published during the preparation of this work that demonstrated that even the induction of robust tolerance using chimerism induction strategies may not always be sufficient to prevent chronic rejection.

In this study, we compare the effects of several clinically relevant CB-based tolerance induction regimens to promote cardiac allograft survival, to induce donor-specific tolerance, and to prevent acute and chronic allograft rejection. We find that treatment regimens consisting of CB alone (CTLA4-Ig and anti-CD40L), CB and donor BMCs, and CB and donor splenocytes (DST) promote long-term allograft survival, but do not confer robust tolerance nor prevent chronic rejection in the face of a rechallenge with a donor skin graft. In contrast, a regimen consisting of CTLA4-Ig, anti-CD40L, donor BMCs, and a minimally myelosuppressive dose of Bu produced stable donor-specific tolerance, and prevented both early and late cellular infiltration and chronic allograft vasculopathy, despite the rigorous rechallenge of a donor skin graft.

## Materials and Methods

### Mice

Adult male 6- to 8-wk-old C57BL/6 (B6) (H-2<sup>b</sup>), BALB/c (H-2<sup>d</sup>), and C3H/HeJ (H-2<sup>k</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in specific pathogen-free conditions and in accordance with institutional guidelines.

### Bone marrow preparation and treatment regimens

Bone marrow was flushed from tibiae, femurs, and humeri. Red cell lysis was performed using a Trizma base ammonium chloride solution. The donor BMCs were resuspended at  $2 \times 10^7$  cells/250  $\mu$ l sterile saline and injected i.v. on the day of cardiac transplantation (day 0). Hamster anti-mouse CD40L (MR1; Bioexpress, Lebanon, NH) and CTLA4-Ig (Bristol-Myers Squibb, Princeton, NJ) were administered on days 0, 2, 4, 6, 14, 28, 60, and 90 (500  $\mu$ g/dose i.p., respectively). The recipients received Bu (20 mg/kg, i.p.) on day 5 and second dose of donor BMCs (i.v.) on day 6 (20). Mice in other groups received CB consisting of anti-mouse CD40L and CTLA4-Ig alone, CB with two doses of donor BMCs (without Bu), or CB with two doses of DST ( $2 \times 10^7$  cells on days 0 and 6).

### Heart grafting

Fully vascularized heterotopic hearts from BALB/c donors were transplanted into the abdomen of B6 recipients using microsurgical technique on day 0, as previously described (21). Graft survival was followed by palpation at least three times per week. Rejection was defined by complete cessation of palpable contraction confirmed by direct visualization. Histo-

logical examination was also performed to confirm the condition of the grafts.

### Skin grafting

Full thickness skin grafts ( $\sim 1$  cm<sup>2</sup>) were transplanted on the dorsal thorax of recipient mice and secured with a Band-Aid for 7 days. Rejection was defined as the complete loss of viable epidermal graft tissue.

### Flow cytometric analysis

We performed multicolor flow cytometry. Donor and recipient cells were distinguished by staining with anti-H-2K<sup>d</sup> and anti-H-2K<sup>b</sup>, respectively. To analyze the degree and distribution of hemopoietic chimerism in multiple compartments and in multiple organs in the recipient, lineage-specific markers such as anti-B220 (for B cells), anti-CD4, anti-Gr1 (for granulocytes), anti-CD11b (for monocytes/macrophages), and CD11c (for dendritic cells) (22, 23) were used. Peripheral blood was analyzed by staining with fluorochrome-conjugated Abs (anti-CD11b, anti-Gr1, anti-B220, anti-CD8, anti-H-2K<sup>d</sup>, anti-H-2K<sup>b</sup>, anti-V $\beta$ 11, anti-V $\beta$ 5.1/5.2, anti-V $\beta$ 8.1/8.2 (BD PharMingen, San Diego, CA), anti-CD4 (Caltag Laboratories, Burlingame, CA), or Ig isotype controls (BD PharMingen)), followed by RBC lysis and washing with a whole blood lysis kit (R&D Systems, Minneapolis, MN). Single cell suspensions of spleen, abdominal lymph nodes, thymus, or bone marrow were also analyzed by staining with fluorochrome-conjugated Abs (anti-B220, anti-CD4, anti-CD8, anti-H-2K<sup>d</sup>, anti-H-2K<sup>b</sup>, anti-V $\beta$ 11, anti-V $\beta$ 5.1/5.2, anti-V $\beta$ 8.1/8.2, or Ig isotype controls) after RBC lysis with a Trizma base ammonium chloride solution.

Dendritic cell-enriched populations were prepared as transiently adherent cells from spleen, lymph nodes, thymus, or bone marrow, as previously described (24), and stained with fluorochrome-conjugated Abs (anti-CD11c, anti-H-2K<sup>d</sup>, anti-I-A<sup>d</sup> (BD PharMingen), or Ig isotype controls). Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA).

### Graft histology

Fresh tissues were fixed in 4% paraformaldehyde until processed and embedded in paraffin (Fisher Scientific, Pittsburgh, PA). Five-micron-thick tissue sections were cut on a microtome and stained with H&E or Masson's trichrome, according to standard procedures. To evaluate the degree of chronic rejection, the number of vessels, including coronary arteries and i.m. arterioles affected with obliterative vasculopathy, and the total number of vessels in each section of the histological specimen were counted. Histological specimens were reviewed by a single histologist (H. Xu) blinded to the treatment modality.

### Statistical analysis

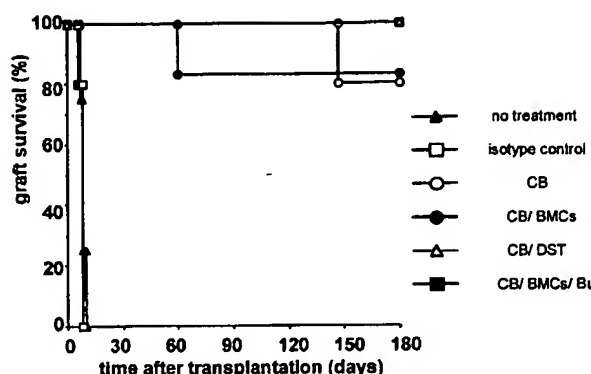
Graft survival between groups was analyzed by Mann-Whitney *U* test using Stat View 5.0 software (Abacus Concepts, Berkeley, CA). Statistical significance of other data was analyzed by unpaired Student's *t* test using Stat View 5.0.

## Results

We have recently reported that a regimen consisting of CB, donor BMCs, and a minimally myelosuppressive, nonablative dose of Bu induces high level mixed hemopoietic chimerism and transplantation tolerance in a rigorous mouse skin graft model (20). The purpose of this study was to compare the effects of this regimen with three putative tolerance induction strategies, including CB alone (anti-CD40L and CTLA4-Ig), CB with BMCs, and CB with DST, on 1) cardiac allograft survival, as assessed by the persistence of a palpable heartbeat; 2) the ability to induce robust tolerance, as assessed by the ability to accept a secondary donor skin graft; 3) the ability to protect allograft from acute and chronic rejection, as assessed by histopathological examination; and 4) the kinetics of the development of hemopoietic chimerism and deletion of donor-reactive T cells, as assessed by flow cytometry.

### Effects on cardiac allograft survival: all CB-based regimens greatly prolong survival of mouse primary cardiac allografts

As seen in Fig. 1, untreated B6 recipients or recipients treated with isotype control Abs rejected BALB/c heart grafts rapidly (median survival time (MST) = 8 days, *n* = 10 and 5, respectively).



**FIGURE 1.** CB with CTLA4-Ig and anti-CD40L induces long-term acceptance of cardiac allografts. B6 (H-2<sup>b</sup>) recipients of BALB/c (H-2<sup>d</sup>) cardiac grafts received CB, BMCs, Bu (■,  $n = 5$ ); CB, DST (△,  $n = 4$ ); CB, BMCs (●,  $n = 5$ ); CB (○,  $n = 6$ ); isotype control (□,  $n = 5$ ); or no treatment (▲,  $n = 10$ ). All animals (B6) received fully allogeneic cardiac grafts (BALB/c) on day 0. Control groups (no treatment, or isotype control) promptly rejected the BALB/c cardiac grafts (MST = 8 days, respectively). Recipients treated with CB-based regimens (i.e., recipients with CB alone; CB and BMCs; CB and DST; or CB, BMCs, and Bu) showed prolonged survival of primary cardiac allografts with MST >180 days, while one recipient each from group with CB alone or with CB and BMCs rejected the grafts on days 147 and 60, respectively. Additional groups performed with these regimens yielded similar survival results, but were harvested for histologic assessment on day 14, 28, 60, or 90.

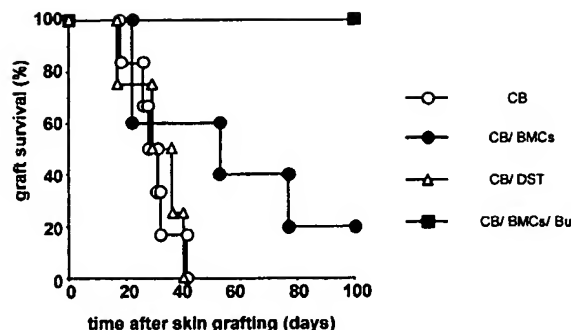
BALB/c hearts were also acutely rejected by B6 recipients receiving only donor BMCs (MST = 9 days,  $n = 6$ ), by those receiving donor BMCs and Bu (MST = 10 days,  $n = 5$ ), and by those treated with Bu only (MST = 10 days,  $n = 4$ ) (data not shown). In contrast, groups treated with CB (i.e., recipients with CB alone ( $n = 6$ ); with CB and BMCs (no Bu) ( $n = 5$ ); with CB and DST ( $n = 4$ ); or with CB, BMCs, and Bu ( $n = 5$ )) showed long-term survival of BALB/c heart grafts (MST >180 days in all these groups) (Fig. 1). There was no statistically significant difference in BALB/c heart survival among these groups.

*Effects on tolerance induction: only recipients of CB, Bu, and donor BMCs develop stable transplantation tolerance*

Next, to determine whether recipients were rendered tolerant, recipients in the various treatment groups with long-term surviving BALB/c heart grafts were rechallenged with secondary skin grafts from BALB/c (donor-specific) and C3H (third party) donors 200 days after primary heart transplantation. Recipients that had received CB, BMCs, and Bu uniformly accepted second BALB/c skin grafts (MST >100 days). In contrast, recipients treated with CB and BMCs, CB and DST, or CB alone showed evidence of donor-specific hyporesponsiveness, but eventually rejected second BALB/c skin grafts (MST = 53, 33, and 30 days;  $n = 4, 4$ , and 5, respectively) (Fig. 2). Recipients from all groups promptly rejected C3H (third party) skin grafts (MSTs = 14 days) (data not shown).

*Effects on acute and chronic rejection—histopathological analysis: CB-based regimens prevent early tissue damage, but only the regimen of CB, Bu, and donor BMCs prevents chronic rejection of heart allografts*

The most commonly used endpoint for the assessment of murine heterotopic cardiac allograft survival is the cessation of a palpable heartbeat. To gain better insight into the degree to which the various regimens protected the allografts from immunologic injury (acute and chronic rejection), we performed detailed histological examinations of these BALB/c heart grafts using H&E and Mas-



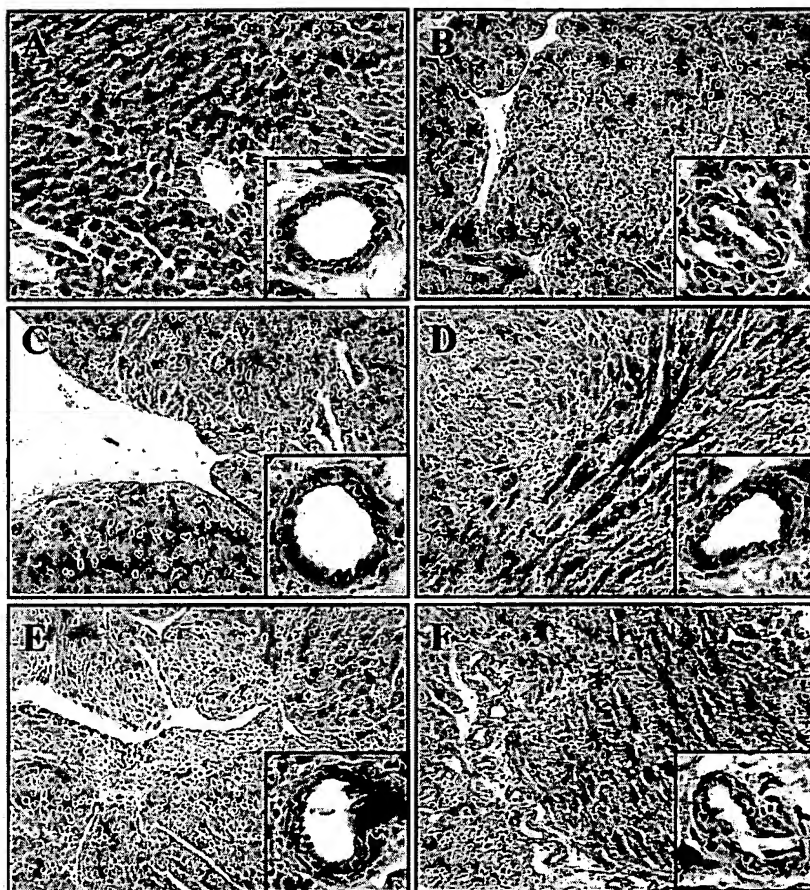
**FIGURE 2.** Only administration of CB, BMCs, and Bu promotes the development of transplantation tolerance in allogeneic recipients. Two hundred days after primary cardiac grafting, recipients with accepted cardiac grafts received secondary donor-specific (BALB/c, H-2<sup>d</sup>) and third party (C3H, H-2<sup>k</sup>) skin grafts. Recipients with CB alone (○,  $n = 5$ ), CB and BMCs (●,  $n = 4$ ), or CB and DST (△,  $n = 4$ ) eventually rejected secondary BALB/c skin grafts with MSTs of 30, 53, and 33 days, respectively. In contrast, BALB/c skin grafts placed on animals treated with CB, BMCs, and Bu (■,  $n = 5$ ) survived indefinitely (>100 days). All recipients rejected third party (C3H) skin grafts within 10–15 days (data not shown).

son's trichrome staining at 14, 28, 60, 90, and 300 (i.e., 100 days after secondary skin grafts) days after heart transplantation or at the time of allograft failure. Allografts harvested at time points up to 90 days posttransplant (while treatment with CB is ongoing) were free of myocardial injury in all groups receiving CB (CB alone (Fig. 3D); CB and BMCs (Fig. 3E); CB and DST (Fig. 3F); or CB, BMCs, and Bu (Fig. 3C)). However, allografts treated with CB alone or CB and DST had rare sparse interstitial infiltrates, whereas allografts treated with CB, BMCs, and Bu were indistinguishable from syngeneic grafts (Fig. 3B). Grafts harvested on day 14, 28, or 60 in these recipients (either treated with CB alone; CB and BMCs; CB and DST; or CB, BMCs, and Bu) had similar histology to those harvested on day 90 (data not shown). These data suggest that prolonged treatment with anti-CD40L and CTLA4-Ig can protect vascularized murine cardiac allografts during the induction phase of these regimens.

The long-term outcomes (300 days) 100 days after rechallenge with a secondary donor skin graft were strikingly different. Allografts in recipients with CB only (Fig. 4D), with CB and BMCs (Fig. 4E), or with CB and DST (Fig. 4F) showed typical changes characteristic of severe chronic rejection, including severe interstitial fibrosis, diffuse infiltration of mononuclear cells, and obliterative vasculopathy with diffuse intimal thickening in coronary arteries in the grafts. The number of coronary arteries and i.m. arterioles affected with obliterative vasculopathy in these three groups ranged from 64 to 87% of vessels observed (Table I). This occurred despite the continued survival of the grafts as assessed by palpation. Examples of normal cardiac histology (Fig. 4A) and BALB/c cardiac allograft undergoing acute rejection in an untreated B6 recipient 10 days after transplantation (Fig. 4B) are shown for comparison.

In contrast, the grafts in mice receiving the chimerism induction regimen of CB, BMCs, and Bu did not show any evidences of either acute or chronic rejection (Fig. 4C). In the recipients of CB, BMCs, and Bu, cardiac allografts were morphological normal and remained free of interstitial or vascular pathology, similar to that of naive BALB/c heart (Fig. 4A). No vessels with obliterative vasculopathy were observed in specimens (10 coronary cross sections/specimen) from recipients treated with CB, BMCs, and Bu (Table

**FIGURE 3.** Morphology of cardiac grafts at day 90. Cardiac grafts were harvested from B6 recipients on day 90 after transplantation. Tissue sections were stained with H&E. *A*, BALB/c naïve heart for comparison. *B*, Syngeneic graft in a B6 recipient on day 90 showing normal heart architecture with little leukocyte infiltration. There is no difference in histology between B6 and BALB/c syngeneic grafts (data not shown). *C*, BALB/c allograft in B6 recipient treated with CB, BMCs, and Bu. *D*, BALB/c allograft in B6 recipient treated with CB alone. *E*, BALB/c allograft in B6 recipient treated with CB and BMCs (no Bu). *F*, BALB/c allograft in B6 recipient treated with CB and DST on day 90. Histology of these grafts (*C–F*) shows that normal heart architecture is preserved. There is no evidence of transplant vasculopathy in coronary vessels in these grafts. However, allografts with CB, BMCs, and Bu have a few infiltrating leukocytes, much like the syngeneic grafts, while allografts with CB alone (*D*) or with CB and DST (*F*) demonstrate somewhat more extensive infiltration in the interstitial space. Similar histological results were obtained from three allografts from each experimental group (H&E; magnification  $\times 200$ ; insets  $\times 400$ ).



1). Very similar results were observed in all groups when recipients were treated with CB on days 0, 2, 4, 6, 14, and 28. We have not tested shorter treatment regimens.

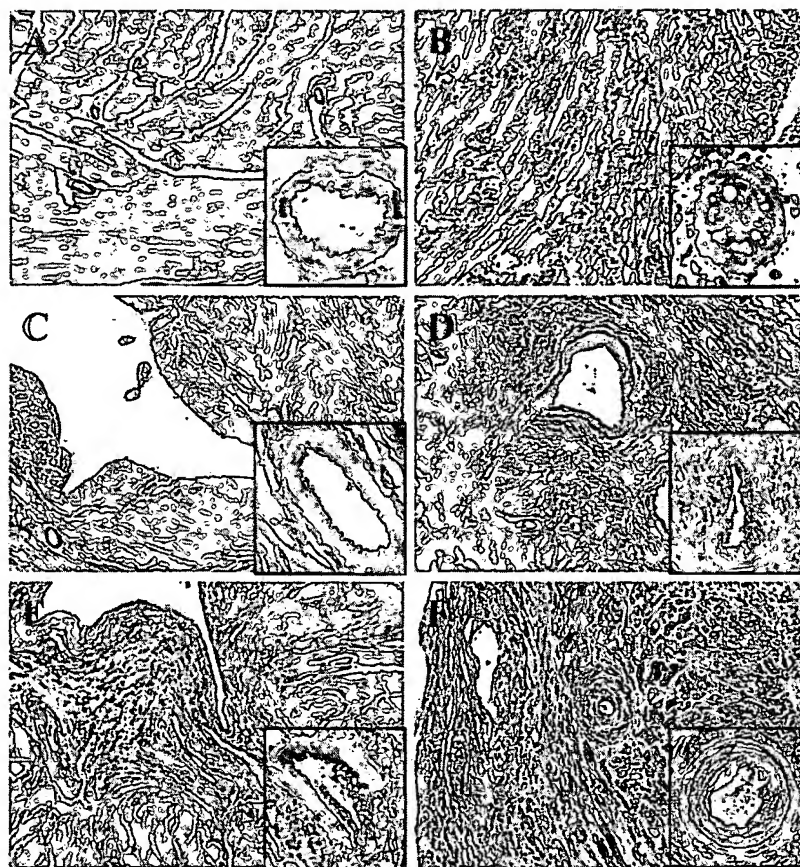
*Effects on recipient immune system: analysis of the distribution and kinetics of hemopoietic chimerism*

Next, we compared the degree and distribution of hemopoietic chimerism in the recipients of each regimen. As expected, only treatment with CB, BMCs, and a nonmyeloablative dose of Bu could induce multilineage hemopoietic chimerism in peripheral blood of recipients after day 14 (Fig. 5, *A–C*). Similar levels of donor chimerism were observed among B220<sup>+</sup>, CD4<sup>+</sup> cells, and CD11c<sup>+</sup> dendritic cells in spleen (Fig. 5*D*), abdominal lymph node (Fig. 5*E*), bone marrow (Fig. 5*F*), and thymus (Fig. 5*G*). Recipients with no treatment, donor BMCs alone, donor BMCs and Bu, or CB alone had no detectable hemopoietic chimerism in any organs (data not shown). Recipients treated with CB and donor BMCs (no Bu), or with CB and two doses of DST showed minimal donor chimerism on day 14 and virtually undetectable levels of donor cells thereafter, suggesting that those donor cells were not engrafted populations, but only passenger leukocytes (Fig. 5, *A* and *B*).

*Effect on recipient immune system: sustained deletion of V $\beta$ 11 and V $\beta$ 5 T cells only occurred with hemopoietic chimerism*

As a measure of the ability of these regimens to reshape the recipients' peripheral T cell repertoire, we tracked the fate of the mouse mammary tumor virus superantigen-reactive T cells as a surrogate marker for antidonor reactive T cells (16, 18, 20). Donor BALB/c mice express mouse mammary tumor virus-8

and 9 in association with MHC class II I-E molecules and delete V $\beta$ 11- and V $\beta$ 5-bearing CD4<sup>+</sup> T cells, whereas recipient B6 mice do not express I-E and use V $\beta$ 11 on  $\sim 4\text{--}5\%$  of CD4<sup>+</sup> T cells and V $\beta$ 5.1/2 on  $\sim 2\text{--}3\%$  of CD4<sup>+</sup> T cells (25, 26). As anticipated, recipients treated with donor BMCs alone, with donor BMCs and Bu, or with Bu alone failed to delete donor-reactive V $\beta$ 11<sup>+</sup> or V $\beta$ 5<sup>+</sup>CD4<sup>+</sup> T cells (data not shown). Recipients treated with CB and BMCs (no Bu) showed a transient decrease in the percentage of CD4<sup>+</sup>V $\beta$ 11<sup>+</sup> and CD4<sup>+</sup>V $\beta$ 5<sup>+</sup> T cells on day 28, but these populations recovered by day 60. This effect was not observed in recipients treated with CB alone (not shown) or CB and DST (Fig. 6, *A* and *B*). In contrast, the recipients with CB, BMCs, and Bu developed profound deletion of CD4<sup>+</sup>V $\beta$ 11<sup>+</sup> and CD4<sup>+</sup>V $\beta$ 5<sup>+</sup> T cells in peripheral blood (Fig. 6*C*), spleen (Fig. 6*D*), and abdominal lymph nodes (Fig. 6*E*). The deletion process with this regimen took place over a surprisingly long period of time. Virtually no peripheral deletion of CD4<sup>+</sup>V $\beta$ 11<sup>+</sup> T cells was evident in blood, spleen, or lymph node at day 14. Only  $\sim 50\%$  depletion was achieved by day 28, and near complete deletion occurred between days 60 and 90. Similar, but slightly more rapid and complete deletion (day 28) of CD4<sup>+</sup>V $\beta$ 5<sup>+</sup> T cells was also observed. Central deletion of CD4<sup>+</sup> (CD8<sup>−</sup>) V $\beta$ 11<sup>+</sup> and CD4<sup>+</sup> (CD8<sup>−</sup>) V $\beta$ 5<sup>+</sup> thymocytes was only observed in the recipients treated with CB, BMCs, and Bu, but this deletion was not apparent until day 60 (Fig. 6*F*). The percentage of V $\beta$ 8-bearing CD4<sup>+</sup> T cells, which are expressed on  $\sim 15\text{--}20\%$  of BALB/c and B6 CD4<sup>+</sup> T cells, was similar in all groups, indicating that the T cell deletion was donor specific in nature (data not shown).



**FIGURE 4.** Morphology of cardiac allografts on day 300 after transplantation. Cardiac allografts that were not rejected 100 days after secondary skin grafting (i.e., the allografts with palpable beating 300 days after primary cardiac transplantation) were harvested from B6 recipients treated with either CB alone; CB and BMCs; CB and DST; or CB, BMCs, and Bu. Tissue sections were stained with Masson's trichrome. *A*, BALB/c naive heart for comparison. *B*, BALB/c allografts in B6 recipients on day 10 without any treatment for comparison. In this allograft, findings are consistent with acute rejection, including diffuse lymphocytic infiltration with myocyte damage. Obliterative vasculopathy was not seen. *C*, BALB/c allograft in B6 recipient treated with CB, BM, and Bu. *D*, BALB/c allograft in B6 recipient treated with CB alone. *E*, BALB/c allograft in B6 recipient treated with CB and BMCs (no Bu). *F*, BALB/c allograft in B6 recipient treated with CB and DST. Histology of allografts in recipients with CB alone, CB and BMCs, or CB and DST (*D–F*) showed extensive fibrosis in the grafts (collagen is highlighted as blue in Masson's trichrome stain), as well as infiltration of mononuclear cells in the interstitial space, especially adjacent to the coronary vessels. Coronary vessels (in insets) showed intimal hyperplasia and resulting narrowing of the lumen, characteristic of the obliterative vasculopathy of chronic rejection. There is no difference in spectrum of vascular pathology in these three groups (*D–F*). In contrast, histology of the allografts in the recipients treated with CB, BMCs, and Bu showed preservation of normal myocyte architecture and normal coronary vessel structure. These grafts were essentially free from any infiltrate and obliterative vasculopathy (*C*). Similar histological results were obtained from three allografts from each experimental group (Masson's trichrome; magnification  $\times 200$ ; insets  $\times 400$ ).

## Discussion

We have recently demonstrated that a regimen consisting of CB, a nonmyeloablative dose of Bu, and donor BMCs induces high level, stable hemopoietic chimerism and robust transplantation tolerance in the mouse skin graft model (20). The skin graft model was used

in these initial studies because it is an immunologically stringent test for the efficacy of this approach. However, unlike organ allografts, the skin model is not primarily vascularized and does not offer a well-characterized method to assess the degree of immunologic injury sustained by the allograft. Given the numerous reports that tolerance protocols can be associated with active immunologic infiltrates (9, 27), and/or transplant vasculopathy, we felt that it was important to determine the ability of this combined CD40/CD28 CB chimerism induction protocol to protect allografts from acute and chronic rejection using the primarily vascularized mouse cardiac allograft model. In addition, we have performed a long-term comparison with other regimens that we and others have described that can also promote long-term survival of primary cardiac allografts.

Not surprisingly, we found no statistically significant difference in primary cardiac graft survival (as assessed by graft palpation) or cardiac allograft histology (before secondary rechallenge of alloantigen) between the regimen inducing mixed chimerism and other regimens including CB alone, CB and BMCs, or CB and

**Table I.** Analysis of the heart grafts 300 days posttransplant<sup>a</sup>

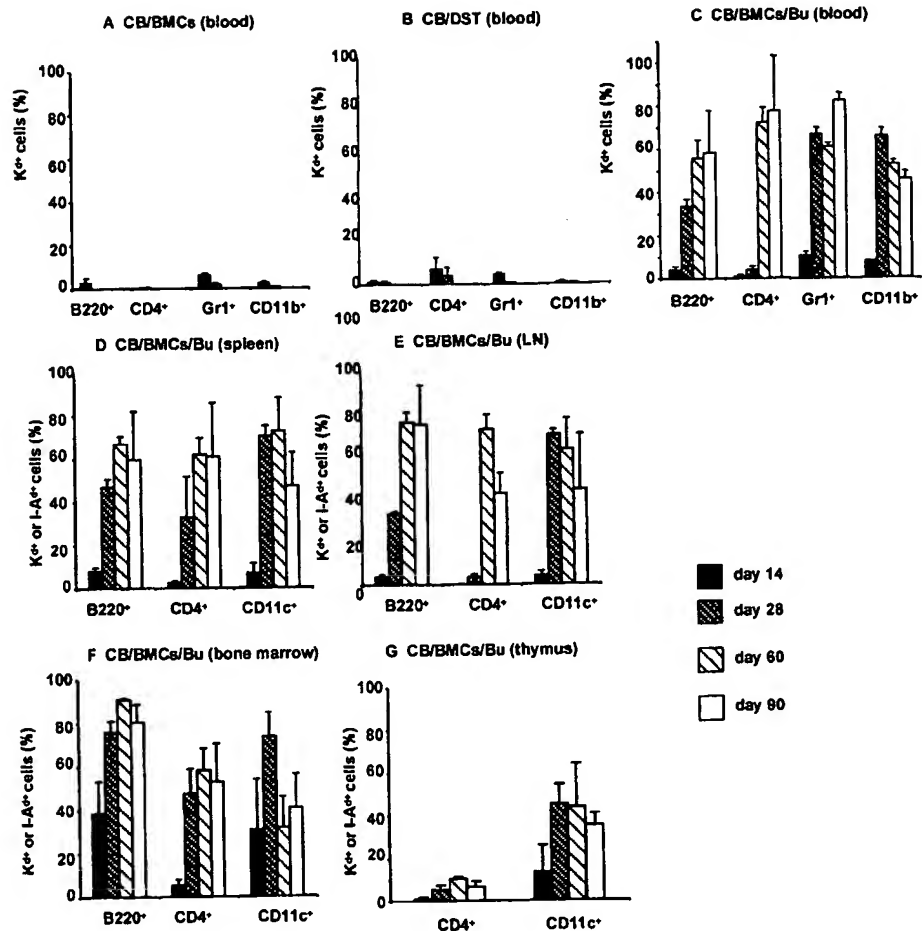
Treatment	No. of Grafts with Vasculopathy	No. of Arteries and Arterioles with Vasculopathy <sup>b</sup>
CB	5/5	9.27 $\pm$ 1.39/14.5 $\pm$ 1.60
CB and BMCs	4/4	10.5 $\pm$ 2.39/13.1 $\pm$ 1.92
CB and DST	4/4	10.8 $\pm$ 1.61/12.4 $\pm$ 2.02
CB, BMCs, and Bu	0/5	0/14.3 $\pm$ 1.46

<sup>a</sup> The number of vessels, including coronary arteries and intramuscular arterioles, with vasculopathy in the heart grafts 300 days after transplantation, was analyzed by a single reviewer blinded to the treatment modality.

<sup>b</sup> The number of the vessels with vasculopathy/the total number of coronary arteries and i.m. arterioles in the graft. The data represent the mean and the SD for each group.



**FIGURE 5.** Degree and distribution of chimerism in peripheral blood, the spleen, abdominal lymph nodes, bone marrow, and the thymus. Donor representation among various hemopoietic lineages (B cell, CD4<sup>+</sup> cell, granulocyte, monocyte/macrophage, and dendritic cell compartments) was determined on days 14, 28, 60, and 90 after primary heart grafting in peripheral blood (A–C), the spleen (D), abdominal lymph nodes (E), the bone marrow (F), and the thymus (G) by flow cytometry. Only treatment with CB, BMCs, and Bu promotes multilineage chimerism in peripheral blood (C), spleen (D), lymph nodes (E), bone marrow (F), and thymus (G), while recipients treated with CB and BMCs (A), or CB and DST (B) showed transient and less than 5% donor cells only detected within 28 days of transplantation. All B6 recipients received fully allogeneic cardiac grafts (BALB/c, H-2<sup>d</sup>) on day 0. Data points represent the mean and the SD for each group ( $n = 3$ ) (B220<sup>+</sup>, B cell compartment; CD4<sup>+</sup>, CD4<sup>+</sup> cell compartment; Gr1<sup>+</sup>, granulocyte compartment; CD11b<sup>+</sup>, monocyte/macrophage compartment; CD11c<sup>+</sup>, dendritic cell compartment).



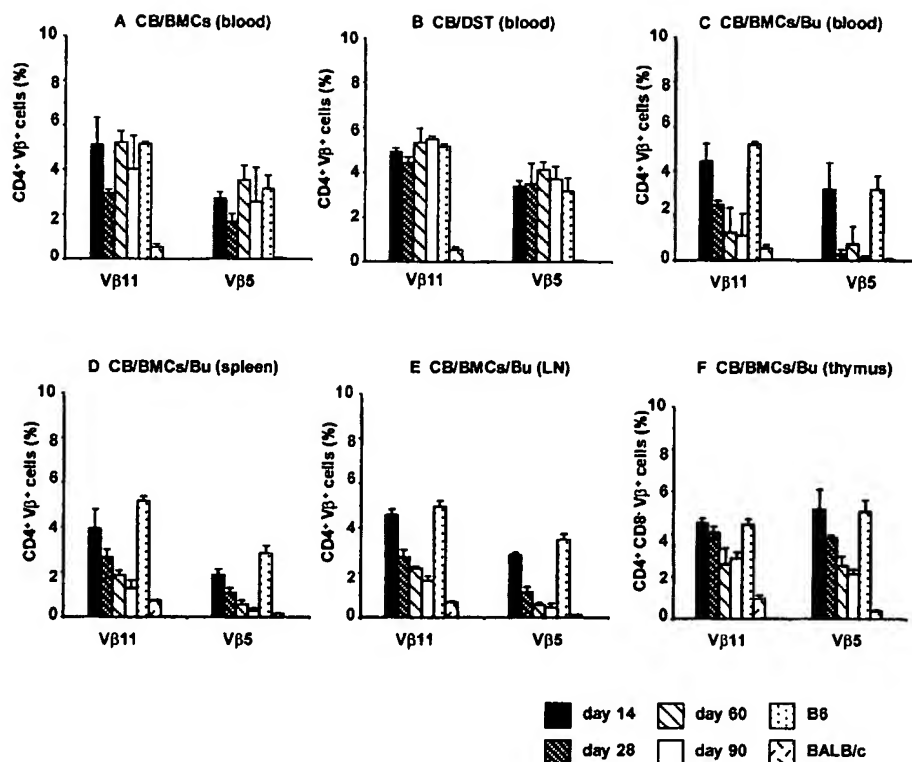
DST (Figs. 1 and 3). For example, on day 90, grafts from all of the groups that received CB continued to beat and showed only rare infiltrating cells equivalent to that observed in syngeneic grafts in the control group. Consistent with our earlier work, and that of others, these data suggest that the combination of CTLA4-Ig and anti-CD40L can protect cardiac allografts from acute rejection for relatively prolonged periods. These data are also important in that they demonstrate that enhanced graft survival that results from this regimen of prolonged CB does not require the presence of a lymphocytic infiltrate of a significant number of regulatory cells. Although this does not exclude a role for a small number of regulatory cells in the graft or a role in the lymphoid tissues, this knowledge is of practical significance for setting expectations for protocol allograft biopsies that might be obtained in the conduct of clinical trials (i.e., an infiltrate cannot be presumed to be an obligatory feature of the enhanced survival of immunotherapies directed at T cell costimulation).

In contrast to the effects on primary graft survival, only the chimerism induction regimen (CB, BMCs, and Bu) prevented cardiac allograft rejection after rechallenge with a secondary donor skin graft. It is reasonable to question the importance of the finding that the protocols differ in their ability to protect from rejection that is only evident after a rechallenge with a donor-specific skin graft, as this scenario would not occur in the clinical setting. However, studies conducted using mice housed in highly protected specific pathogen-free conditions also may not accurately reflect the stability of tolerance under normal external environmental circumstances. For example, it is known that T cells specific for pathogens overlap with the allospecific repertoire (28, 29). In the

specific pathogen-free conditions used for most murine tolerance studies, activation of cross-reactive T cells might not occur often, if at all, whereas in a clinical setting it is possible, if not likely, that immune responses to environmental pathogens might involve alloreactive cells that could precipitate rejection of grafts maintained by the more tenuous mechanisms that sustain allograft survival in the nonchimerism-based regimens tested in this study. It is noteworthy that one recipient in both the groups receiving CB only and the CB and BMCs group rejected BALB/c grafts with histology typical of severe chronic rejection before placement of the secondary skin graft. Additionally, the sustained presence of the vascularized cardiac graft was not sufficient for the induction of robust tolerance that has been observed with other tolerance regimens (10). In contrast, in the chimeric mice receiving CB, BMCs, and Bu, the inability of donor rechallenge to precipitate rejection suggests that viral infections are unlikely to perturb tolerance established by this regimen. Indeed, although graft histology was not assessed in our skin graft studies, acute lymphocytic choriomeningitis virus infection failed to promote overt rejection of skin grafts after the establishment of hemopoietic chimerism (30).

The findings of this study are in apparent conflict with several reports that have suggested that CB or CB with donor cells can prevent chronic rejection (5, 6, 31–33). For example, we have previously reported that simultaneous blockade of CD28 and CD40 pathways inhibits the chronic transplant vasculopathy in BALB/c heart grafts in C3H recipients (5), and other groups have demonstrated that blockade of these pathways inhibits transplant vasculopathy in C57BL/10 aortic grafts in C3H recipients (31), rat kidney (32), and heart allograft (33). In addition to role of the

**FIGURE 6.** Deletion of donor-reacting T cells in peripheral blood, the spleen, abdominal lymph nodes, and the thymus. Deletion of the CD4<sup>+</sup>Vβ11<sup>+</sup>, Vβ5.1/2<sup>+</sup>, and Vβ8.1/2<sup>+</sup> T cells was determined on days 14, 28, 60, and 90 after primary heart grafting in peripheral blood (A–C), the spleen (D), abdominal lymph nodes (E), and the thymus (F) by flow cytometry. Only recipients treated with CB, BMCs, and Bu showed sustained deletion of Vβ11<sup>+</sup> and Vβ5.1/2<sup>+</sup>CD4<sup>+</sup> T cells first evident at day 28 after transplant (C–F). Recipients treated with CB and BMCs (no Bu) showed transient deletion of Vβ11<sup>+</sup>CD4<sup>+</sup> T cells on day 28, but had Vβ11<sup>+</sup> and Vβ5.1/2<sup>+</sup> levels consistent with wild-type B6 levels (4–5% and 2–3%, respectively) after day 60 (A). Other recipients did not show any deletion of Vβ11<sup>+</sup> and Vβ5.1/2<sup>+</sup>CD4<sup>+</sup> T cells. Vβ8.1/2<sup>+</sup>CD4<sup>+</sup> T cells were not deleted by any group (data not shown). Similar results have been observed in 100 mice from multiple experiments. Data points represent the mean and SD for each group (*n* = 3).



secondary skin graft discussed above, there are several other possible reasons for this apparent discrepancy. The strain combination in the current study (B6 recipients and BALB/c donors) has been demonstrated to be vigorous in its ability to overcome the effect of CB, and thus provides a more challenging barrier for the induction of tolerance (34, 35). Second, in the current study, the histological examination of the heart grafts was performed at 300 days after transplant (100 days after rechallenge with a donor skin graft). This is a considerably longer follow-up than in the earlier reports. Third, there are several subtle differences in the treatment regimens between studies, such as the source and timing of blockade or donor cell administration relative to transplantation of the heart graft, that may have contributed to the different outcomes.

Recently, Russell et al. (4) have reported that successful chimerism induction strategies may, in certain circumstances, induce tolerance, but not prevent chronic rejection. In their studies, mixed chimerism was induced in B6 recipients from B10.A donors using a regimen consisting of total body irradiation, depletion of CD4 and CD8 cells and a single dose anti-mouse CD40L, followed by confirmation of tolerance by a challenge of donor-specific skin graft. Afterward, heart allografts were transplanted into the recipients with no further immunotherapy. Chronic vascular rejection was observed in allografts transplanted to these chimeric recipients. There are several possibilities for different results between Russell's and ours. First, it is possible that immune responses to heart-specific polymorphic Ag(s) might play a role in the rejection observed in their experiments. In our experiments, the induction of mixed chimerism and transplantation of the heart were performed simultaneously under the cover of CB, while in Russell's experiments mixed chimerism was established at least 100 days before heart transplantation. Thus, it is possible that transplantation of the heart under the cover of CB might tolerize Ag-specific T cells to heart-specific polymorphic Ag(s) not expressed by the bone marrow, whereas in the Russell experiments delayed heart transplantation would expose the recipient to these putative Ags in the

absence of any immunosuppression, perhaps contributing to the observed vasculopathy. Second, not only alloantigen-dependent, but also alloantigen-independent factors can cause chronic rejection (36). Previous reports have suggested that cold ischemia/reperfusion injury can provoke chronic organ dysfunction and vascular remodeling and that administration of CTLA4-Ig can prevent these lesions (37, 38). Thus, it is possible that in our experiments CB could protect the heart graft from not only T cell-mediated responses, but also inhibit ischemia/reperfusion injury via mechanisms that are not completely understood. Third, as suggested by Russell's group, NK cells might be involved in the pathogenesis of obliterative vasculopathy in cardiac allografts. Several studies have provided evidence that the CD40/CD154 and CD28/B7 pathways may play an important role in the activation of NK cells (39). However, the recent report that NK cells play an important role in cardiac allograft rejection in CD28<sup>-/-</sup> mice suggests that blockade of this pathway alone is insufficient to inhibit NK-induced rejection. Thus, the incorporation of agents to block both the CD40 and CD28 pathways in the peritransplant period in our studies may synergistically inhibit NK cell-mediated injury that contributes to chronic vasculopathy. Finally, the duration of CB in our studies was considerably longer than in most earlier studies. Given the surprisingly long period of time (~60 days) to promote complete deletion of donor-reactive T cells, it is possible that prolonged CB plays an important role by protecting the allograft until the deletion process is complete.

In summary, the data presented have shown that several regimens incorporating CD28 and CD40 blockade with donor cell infusions prolong cardiac allograft survival and protect the allografts from peritransplant infiltration and immunologic injury. However, we have found that only a regimen consisting of CD28 and CD40 blockade together with a minimally myelosuppressive dose of Bu and donor BMCs promoted robust deletional tolerance. Importantly, this regimen also prevented the development of chronic



allograft vasculopathy. These data suggest that further development of regimens targeting the CD40 and CD28 pathways to promote chimerism and tolerance may have a significant impact on chronic rejection, an untreatable cause of clinical transplant failure.

## References

- Denton, M. D., C. C. Magee, and M. H. Sayegh. 1999. Immunosuppressive strategies in transplantation. *Lancet* 353:1083.
- Libby, P., and J. S. Pober. 2001. Chronic rejection. *Immunity* 14:387.
- Shimizu, K., U. Schonbeck, F. Mach, P. Libby, and R. N. Mitchell. 2000. Host CD40 ligand deficiency induces long-term allograft survival and donor-specific tolerance in mouse cardiac transplantation but does not prevent graft arteriosclerosis. *J. Immunol.* 165:3506.
- Russell, P. S., C. M. Chase, M. Sykes, H. Ito, J. Shaffer, and R. B. Colvin. 2001. Tolerance, mixed chimerism, and chronic transplant arteriopathy. *J. Immunol.* 167:5731.
- Larsen, C. P., E. T. Elwood, D. Z. Alexander, S. C. Ritchie, R. Hendrix, C. Tucker-Burden, H. R. Cho, A. Aruffo, D. Hollenbaugh, P. S. Linsley, et al. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381:434.
- Sayegh, M. H., X. G. Zheng, C. Magee, W. W. Hancock, and L. A. Turka. 1997. Donor antigen is necessary for the prevention of chronic rejection in CTLA4Ig-treated murine cardiac allograft recipients. *Transplantation* 64:1646.
- Judge, T. A., Z. Wu, X. G. Zheng, A. H. Sharpe, M. H. Sayegh, and L. A. Turka. 1999. The role of CD80, CD86, and CTLA4 in alloimmune responses and the induction of long-term allograft survival. *J. Immunol.* 162:1947.
- Markees, T. G., N. E. Phillips, E. J. Gordon, R. J. Noelle, L. D. Shultz, J. P. Mordes, D. L. Greiner, and A. A. Rossini. 1998. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4<sup>+</sup> T cells, interferon- $\gamma$ , and CTLA4. *J. Clin. Invest.* 101:2446.
- Kirk, A. D., D. M. Harlan, N. N. Armstrong, T. A. Davis, Y. Dong, G. S. Gray, X. Hong, D. Thomas, J. H. Fechner, Jr., and S. J. Knechtle. 1997. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc. Natl. Acad. Sci. USA* 94:8789.
- Hamano, K., M. A. Rawsthorne, A. R. Bushell, P. J. Morris, and K. J. Wood. 1996. Evidence that the continued presence of the organ graft and not peripheral donor microchimerism is essential for maintenance of tolerance to alloantigen in vivo in anti-CD4 treated recipients. *Transplantation* 62:856.
- Billingham, R. E., L. Brent, and P. B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature* 172:603.
- Owen, R. D. 1945. Immunogenetic consequence of vascular anastomosis between bovine twins. *Science* 102:400.
- Ildstad, S. T., and D. H. Sachs. 1984. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* 307:168.
- Sharabi, Y., and D. H. Sachs. 1989. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J. Exp. Med.* 169:493.
- Tomita, Y., A. Khan, and M. Sykes. 1994. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a nonmyeloablative regimen. *J. Immunol.* 153:1087.
- Wekerle, T., M. H. Sayegh, J. Hill, Y. Zhao, A. Chandraker, K. G. Swenson, G. Zhao, and M. Sykes. 1998. Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *J. Exp. Med.* 187:2037.
- Quesenberry, P. J., S. Zhong, H. Wang, and M. Stewart. 2001. Allogeneic chimerism with low-dose irradiation, antigen presensitization, and costimulator blockade in H-2 mismatched mice. *Blood* 97:557.
- Durham, M. M., A. W. Bingaman, A. B. Adams, J. Ha, S. Y. Waitze, T. C. Pearson, and C. P. Larsen. 2000. Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *J. Immunol.* 165:1.
- Wekerle, T., J. Kurtz, H. Ito, J. V. Ronquillo, V. Dong, G. Zhao, J. Shaffer, M. H. Sayegh, and M. Sykes. 2000. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat. Med.* 6:464.
- Adams, A. B., M. M. Durham, L. Kean, N. Shirasugi, J. Ha, M. A. Williams, P. A. Rees, M. C. Cheung, S. Mittelstaedt, A. W. Bingaman, et al. 2001. Costimulation blockade, busulfan, and bone marrow promote titratable macrochimerism, induce transplantation tolerance, and correct genetic hemoglobinopathies with minimal myelosuppression. *J. Immunol.* 167:1103.
- Shirasugi, N., Y. Ikeda, Y. Akiyama, K. Matsumoto, K. Hamano, K. Esato, H. Bashuda, H. Yagita, K. Okumura, H. Takami, et al. 2001. Induction of hyporesponsiveness to fully allogeneic cardiac grafts by intratracheal delivery of alloantigen. *Transplantation* 71:561.
- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
- Fukao, T., S. Matsuda, and S. Koyasu. 2000. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN- $\gamma$  production by dendritic cells. *J. Immunol.* 164:64.
- Larsen, C. P., S. C. Ritchie, R. Hendrix, P. S. Linsley, K. S. Hathcock, R. J. Hodes, R. P. Lowry, and T. C. Pearson. 1994. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. *J. Immunol.* 152:5208.
- Bill, J., O. Kanagawa, D. L. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of V $\beta$ 11-bearing T cells. *J. Exp. Med.* 169:1405.
- Dyson, P. J., A. M. Knight, S. Fairchild, E. Simpson, and K. Tomonari. 1991. Genes encoding ligands for deletion of V $\beta$ 11 T cells cosegregate with mammary tumor virus genomes. *Nature* 349:531.
- O'Connell, P. J., A. Mba-Jonas, G. E. Levenson, D. M. Heisey, K. C. Meyer, R. B. Love, and W. J. Burlingham. 1998. Stable lung allograft outcome correlates with the presence of intra-graft donor-derived leukocytes. *Transplantation* 66:1167.
- Welsh, R. M., T. G. Markes, B. A. Woda, K. A. Danicls, M. A. Brehm, J. P. Mordes, D. L. Greiner, and A. A. Rossini. 2000. Virus-induced abrogation of transplantation tolerance induced by donor-specific transfusion and anti-CD154 antibody. *J. Virol.* 74:2210.
- Burrows, S. R., S. L. Silins, R. Khanna, J. M. Burrows, M. Rischmueller, J. McCluskey, and D. J. Moss. 1997. Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. *Eur. J. Immunol.* 27:1726.
- Williams, M. A., J. T. Tan, A. B. Adams, M. M. Durham, N. Shirasugi, J. K. Whitmire, L. E. Harrington, R. Ahmed, T. C. Pearson, and C. P. Larsen. 2001. Characterization of virus-mediated inhibition of mixed chimerism and allospecific tolerance. *J. Immunol.* 167:4987.
- Sun, H., V. Subbotin, C. Chen, A. Aitouch, L. A. Valdivia, M. H. Sayegh, P. S. Linsley, J. J. Fung, T. E. Starzl, and A. S. Rao. 1997. Prevention of chronic rejection in mouse aortic allografts by combined treatment with CTLA4-Ig and anti-CD40 ligand monoclonal antibody. *Transplantation* 64:1838.
- Chandraker, A., H. Azuma, K. Nadeau, C. B. Carpenter, N. L. Tilney, W. W. Hancock, and M. H. Sayegh. 1998. Late blockade of T cell costimulation interrupts progression of experimental chronic allograft rejection. *J. Clin. Invest.* 101:2009.
- Kim, K. S., M. D. Denton, A. Chandraker, A. Knoflach, R. Milord, A. M. Waaga, L. A. Turka, M. E. Russell, R. Peach, and M. H. Sayegh. 2001. CD28-B7-mediated T cell costimulation in chronic cardiac allograft rejection: differential role of B7-1 in initiation versus progression of graft arteriosclerosis. *Am. J. Pathol.* 158:977.
- Trambley, J., A. W. Bingaman, A. Lin, E. T. Elwood, S. Y. Waitze, J. Ha, M. M. Durham, M. Corbascio, S. R. Cowan, T. C. Pearson, and C. P. Larsen. 1999. Asialo GM1<sup>+</sup> CD8<sup>+</sup> T cells play a critical role in costimulation blockade-resistant allograft rejection. *J. Clin. Invest.* 104:1715.
- Williams, M. A., J. Trambley, J. Ha, A. B. Adams, M. M. Durham, P. Rees, S. R. Cowan, T. C. Pearson, and C. P. Larsen. 2000. Genetic characterization of strain differences in the ability to mediate CD40/CD28-independent rejection of skin allografts. *J. Immunol.* 165:6849.
- Tullius, S. G., and N. L. Tilney. 1995. Both alloantigen-dependent and -independent factors influence chronic allograft rejection. *Transplantation* 59:313.
- Takada, M., A. Chandraker, K. C. Nadeau, M. H. Sayegh, and N. L. Tilney. 1997. The role of the B7 costimulatory pathway in experimental cold ischemia/reperfusion injury. *J. Clin. Invest.* 100:1199.
- Chandraker, A., M. Takada, K. C. Nadeau, R. Peach, N. L. Tilney, and M. H. Sayegh. 1997. CD28-B7 blockade in organ dysfunction secondary to cold ischemia/reperfusion injury. *Kidney Int.* 52:1678.
- Carbone, E., G. Ruggiero, G. Terrazano, C. Palomba, C. Manzo, S. Fontana, H. Spijs, K. Karre, and S. Zappacosta. 1997. A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction. *J. Exp. Med.* 185:2053.